

Antibody mediated *in vivo* delivery of small interfering RNAs via cell-surface receptors

Erwei Song¹, Pengcheng Zhu¹, Sang-Kyung Lee, Dipanjan Chowdhury, Steven Kussman, Derek M Dykxhoorn, Yi Feng, Deborah Palliser, David B Weiner, Premrata Shankar, Wayne A Marasco & Judy Lieberman

Delivery of small interfering RNAs (siRNAs) into cells is a key obstacle to their therapeutic application. We designed a protamine-antibody fusion protein to deliver siRNA to HIV-infected or envelope-transfected cells. The fusion protein (F105-P) was designed with the protamine coding sequence linked to the C terminus of the heavy chain Fab fragment of an HIV-1 envelope antibody. siRNAs bound to F105-P induced silencing only in cells expressing HIV-7 envelope. Additionally, siRNAs targeted against a core component of HIV-1 replication, *gag*, inhibited HIV replication in hard-to-transfect, HIV-infected primary T cells. Intratumoral or intravenous injection of F105-P-complexed siRNAs into mice targeted HIV envelope-expressing B16 melanoma cells, but not normal tissue or envelope-negative B16 cells; injection of F105-P with siRNAs targeting *c-myc*, *MDM2* and *VEGF* inhibited envelope-expressing subcutaneous B16 tumors. Furthermore, an ErbB2 single-chain antibody fused with protamine delivered siRNAs specifically into ErbB2-expressing cancer cells, thereby demonstrating the potential for systemic, cell-type specific, antibody-mediated siRNA delivery.

There is increasing enthusiasm for developing therapies based on RNA interference (RNAi)—post-transcriptional gene silencing mediated by small RNA duplexes of 19–23 base pairs (bp). The advantage of RNAi lies in its high specificity and potent gene silencing, coupled with the fact that it can potentially target every gene and be processed by every cell (reviewed in ref. 1). Although some questions remain about specificity and activation of off-target effects^{2–4}, none of these problems has yet been documented *in vivo*. Moreover, some potential untoward events can likely be avoided by judicious choice of sequences or chemical modification of siRNAs.

The main obstacle to developing siRNA as a small-molecule drug is delivering it *in vivo* across the cell membrane to the cytoplasm where it can enter the RNAi pathway and guide the sequence-specific mRNA degradation. In the absence of transfection reagents or high pressure that may damage the plasma membrane, most cells, including cells that actively sample their environment, such as macrophages, do not take up siRNAs. An exception may be pulmonary epithelial cells, since protection against respiratory syncytial virus infection in the lung was achieved in one report by nasal administration of siRNAs without any transfection reagent⁵. Early studies validating the therapeutic potential of siRNAs in mice used high-pressure (so-called hydrodynamic) intravenous injection to force siRNAs into cells⁶. However, hydrodynamic injection, which causes right-sided heart failure, is not practical for systemic human use. Although transfection can deliver siRNAs locally, a systemic method to deliver siRNAs to specific cells via cell-surface receptors would provide a means to introduce siRNAs into desired cells to

achieve maximal therapeutic benefit, decrease the amount of drug required and avoid nonspecific silencing and toxicity in bystander cells.

We took advantage of the nucleic acid-binding properties of protamine, which nucleates DNA in sperm, to deliver siRNAs via an antibody Fab fragment-protamine fusion protein⁷. The Fab fragment was used to avoid potential side effects from interactions of complement and other molecules with the antibody constant region. As a proof of concept, a Fab antibody fragment directed against HIV-7 envelope (F105) fused to protamine was used to deliver siRNAs and silence gene expression specifically in HIV-infected cells or cells transfected to express HIV envelope glycoprotein gp120 (HIV env). F105-P was previously shown to carry plasmid DNA into HIV-infected cells^{7,8}. siRNAs bound to the fusion protein and did not require covalent coupling for effective delivery. The strategy was effective at delivering siRNAs into primary cells, such as T lymphocytes, which are highly resistant to transfection. Using B16 melanoma cells transfected with an expression vector for HIV *env*, intravenous or intratumoral injection of F105-P-complexed siRNAs delivered siRNAs only into *env*-expressing (*env*⁺) tumors, but not into normal tissues or *env*-negative tumors, and inhibited tumor outgrowth when the siRNAs targeted oncogenes. This method can be generalized since we could use an anti-ErbB2-protamine fusion protein to deliver siRNAs specifically to ErbB2-expressing (ErbB2⁺) breast cancer cells. This fusion protein used a single-chain antibody expressed from baculovirus in insect cells, which may be a more efficient expression system.

CBR Institute for Biomedical Research and Department of Pediatrics, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, USA. Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA. Dana Farber Cancer Institute and Department of Medicine, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115, USA. ¹These authors contributed equally to this work. Correspondence should be addressed to J.L. (lieberman@cbr.med.harvard.edu).

Published online XX XX XXXX; doi:10.1038/nbtXXXX

RESULTS

F105-P delivers siRNAs only to HIV env⁺ cells

F105-P was expressed and purified from COS cells transfected with a bicistronic plasmid encoding both the F105 Igk light chain and the heavy chain Fab fragment fused at its C terminus to protamine. To determine to what extent F105-P binds siRNA, we incubated a fixed amount of fluorescein isothiocyanate (FITC)-labeled siRNA with varying amounts of the fusion protein, precipitated it with anti-protamine coupled beads and measured the absorbance of captured siRNA at 488 nm. We found that each molecule of F105-P can bind ~6 siRNA molecules (Fig. 1a). To determine whether F105-P was able to deliver siRNA specifically into cells expressing HIV env, FITC-siRNA was added either alone or with the unmodified F105 antibody or with F105-P to an HIV-infected Jurkat cell culture. Lipid transfection was used as a positive control for delivery. Transfected FITC-siRNA was comparably taken up by ~70% of both uninfected and infected cells. Neither infected nor uninfected cells appreciably took up FITC-siRNA by themselves or when mixed with the antibody fragment lacking protamine, with an irrelevant antibody-protamine fusion protein or with unmodified protamine. When mixed with F105-P, the uninfected cells still did not take up the siRNA, whereas

40% of the infected Jurkat cells did (Fig. 1b). Specific delivery of *gag* siRNA into cells expressing HIV env was verified by modified northern blot analysis using mouse melanoma B16 cells stably transfected with envelope glycoprotein 160 (gp160-B16) or empty vector (B16) (Fig. 1c). *gag* siRNA was detected in gp160-B16 cells treated with *gag* siRNA mixed with F105-P, but not when gp160-negative B16 cells were incubated with the same mixture or in gp160-B16 cells incubated with uncomplexed *gag*-siRNA. We found that siRNA uptake plateaued when about 100 pmol [100 nM] was added.

siRNA delivered by F105-P silences gene expression

To evaluate whether F105-P-delivered siRNA can silence target gene expression, we used F105-P to introduce siRNAs targeting *EGFP* into HeLa cells stably expressing *EGFP* (HeLa-GFP). HeLa-GFP cells were transfected by HIV λ HXB3 with about 80% efficiency. *EGFP* siRNA delivered with F105-P reduced *EGFP* mRNA (Fig. 2a) and protein (Fig. 2b) in a dose-dependent manner only in cells that stained for HIV *gag* antigen. Silencing plateaued at about 300 pmol [300 nM] of siRNA. Silencing by F105-P-delivered siRNA was specific as no reduction of *EGFP* expression was observed in untransfected p24⁺ cells, with irrelevant *fas* siRNA, without antibody or with

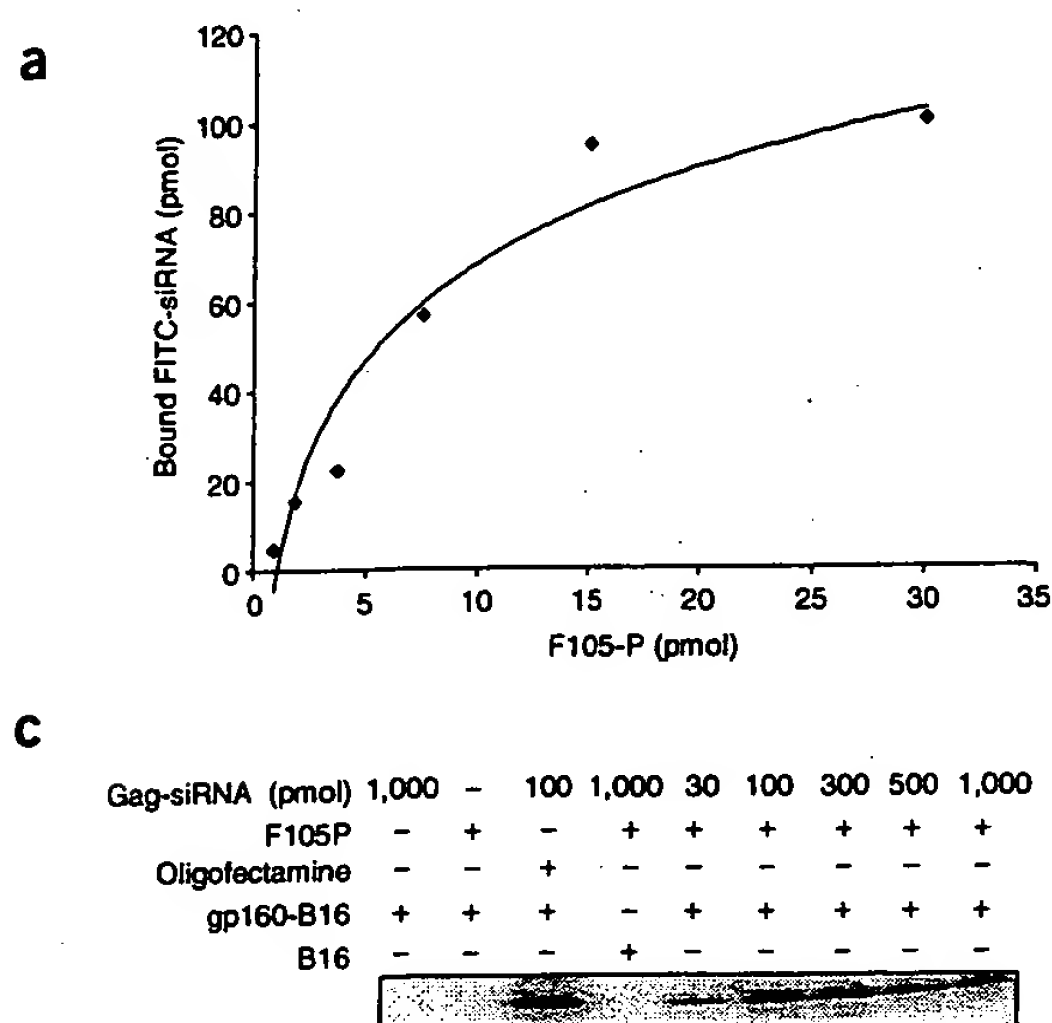
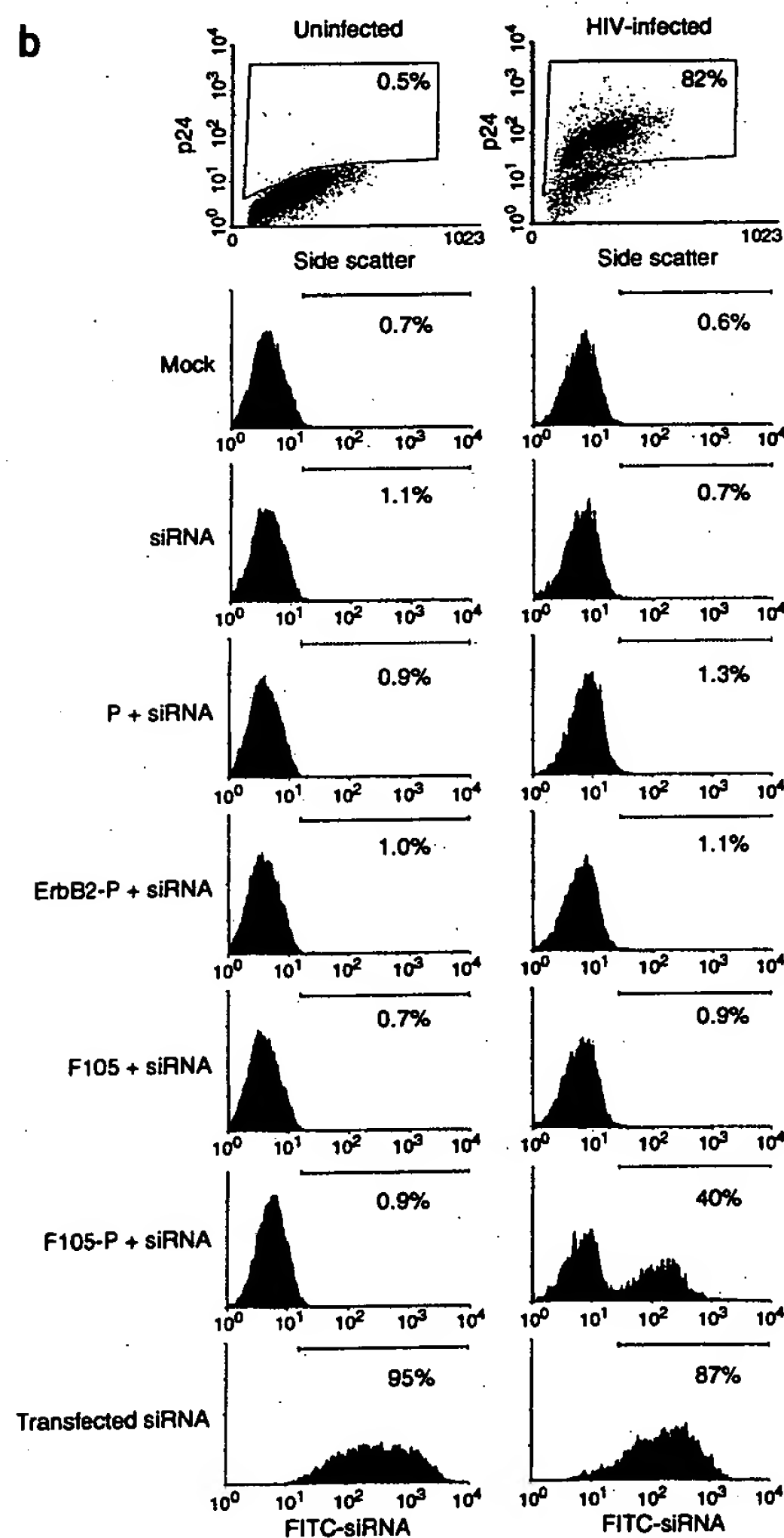


Figure 1 F105-P binds and delivers siRNAs only into HIV env-expressing cells. (a) Each F105-P molecule can bind approximately six FITC-labeled siRNA molecules. A fixed amount of FITC-siRNA was incubated with varying amounts of F105-P bound to anti-protamine-coupled beads and binding of bead-bound FITC-siRNA measured by fluorescence intensity compared to a standard curve. (b) F105-P delivers FITC-labeled siRNA only into HIV-infected Jurkat cells. Jurkat cells were either uninfected (top row, left) or infected (top row, right) with HIV IIIB. About 82% of cells became productively infected as assessed by intracellular staining for HIV p24. HIV env antibody coupled to protamine (F105-P), but not uncoupled antibody (F105), protamine (P), irrelevant ErbB2 single-chain antibody coupled to protamine (ErbB2-P) or medium alone, delivers FITC-labeled siRNA only into gated infected Jurkat cells. Approximately 40% of the HIV-infected cells took up FITC-labeled siRNA, whereas the uninfected cells did not. There was no difference in uptake between infected and uninfected cells when the siRNAs were transfected. (c) F105-P delivers *gag* siRNA into HIV env-expressing gp160-B16 cells, but not into env-negative B16 cells. Cells were analyzed 2 d after treatment by modified northern blot probed with *gag* siRNA antisense strand. Transfected cells serve as a positive control for delivery.



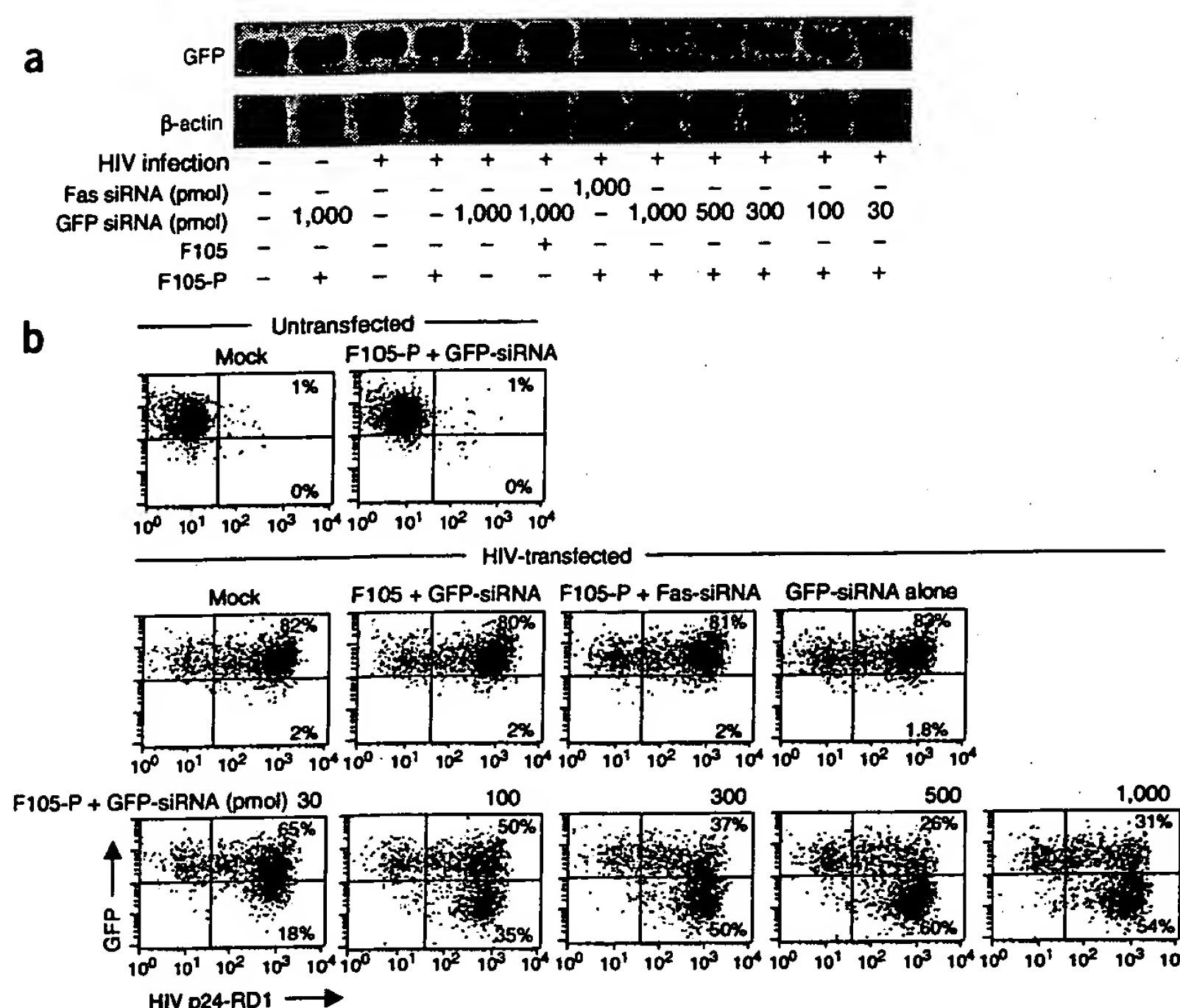


Figure 2 GFP-siRNA delivered by F105-P reduces EGFP expression only in HeLa-GFP cells transfected with HIV λ HXB3. (a,b) HeLa-GFP cells that were transfected with HIV plasmid with ~80% efficiency or mock transfected were treated with GFP siRNA or fas siRNA delivered by F105 or F105-P or medium and analyzed for EGFP mRNA (a) by northern blot or protein (b) by flow cytometry. EGFP expression is silenced when GFP siRNA is complexed with F105-P only in infected cells, which stain for intracellular HIV p24. The p24- untransfected cells in each culture have not down-modulated EGFP and serve as an internal specificity control. Silencing increases in a dose-dependent manner.

unmodified F105 in place of F105-P. The reduction in EGFP mRNA confirms that siRNA delivered by the fusion protein silences target gene expression via mRNA degradation.

siRNA delivered by F105-P inhibits HIV in infected T cells

Primary T cells are notoriously difficult to transfect with conventional lipid-based strategies. We therefore evaluated whether F105-P could deliver gag siRNA into HIV-infected CD4 T cells to reduce virus replication. F105-P loaded with gag siRNA reduced HIV replication in previously infected CD4 T cells in a dose-dependent manner (Fig. 3a). The proportion of productively infected cells declined from 85% in untreated cultures to 36% when 1 nM of gag siRNA was added. Moreover, even with tenfold less siRNA, the proportion of cells staining for HIV gag was only 45%. Release of viral particles from F105-P and gag siRNA-treated primary cells into culture supernatants, as measured by enzyme-linked immunosorbent assay (ELISA) for HIV

p24, was reduced from 170 ng/ml to <40 ng/ml when ≥ 100 pmol of siRNA was used (Fig. 3b). In these experiments, infection was reduced by ~30% (from ~170 ng/ml to ~115 ng/ml) in the presence of either F105 or F105-P antibody alone, owing to the neutralizing activity of the antibody. Nonetheless, the siRNA-coated antibody more efficiently suppressed HIV replication in these difficult-to-transduce primary T cells. Moreover, suppression could be achieved even in cells that were already productively infected.

Oncogene siRNAs delivered by F105-P inhibit proliferation

Because there is no good mouse model for HIV, we subcutaneously injected gp160-B16 cells as a tumor model, to test the ability of antibody-protamine fusion proteins to deliver siRNAs *in vivo*. siRNAs targeting a variety of oncogenes, tumor growth factors, anti-apoptotic genes and drug resistance genes have been shown to suppress tumor growth, mostly *in vitro*¹. If siRNAs could be specifically targeted to

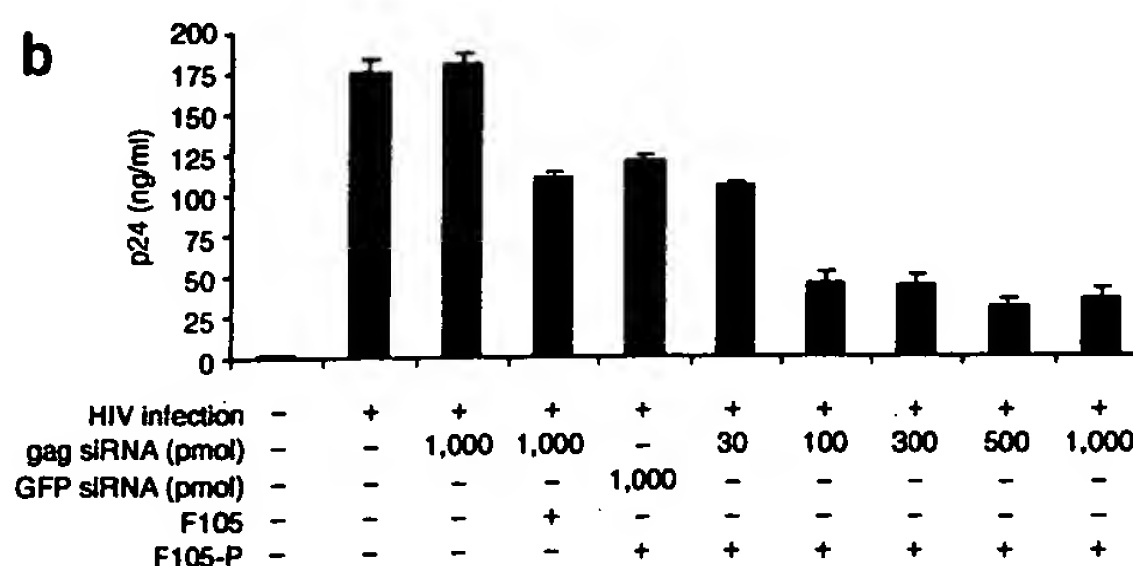
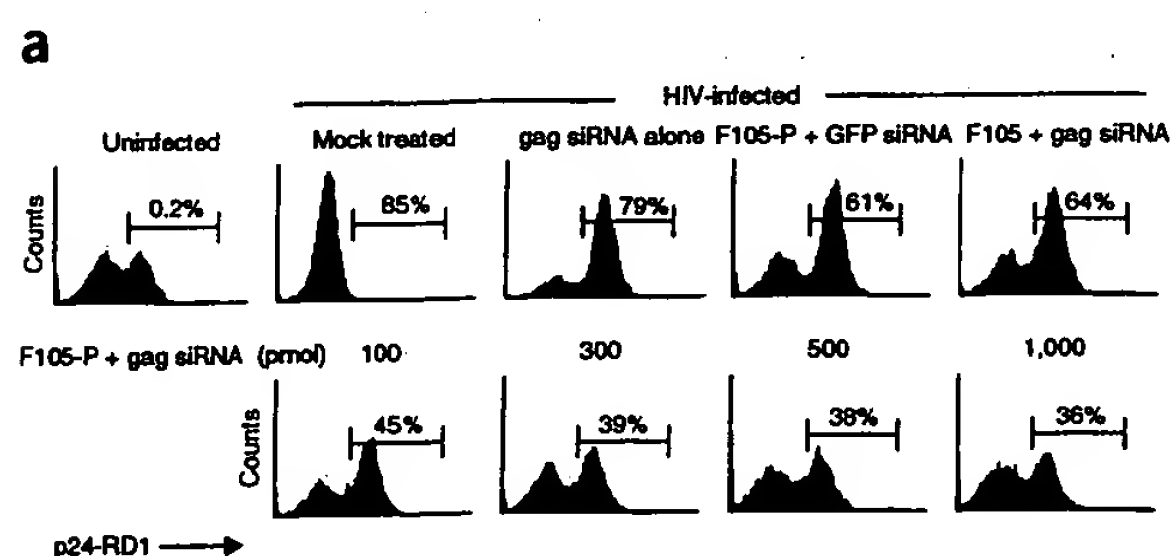


Figure 3 F105-P complexed with gag siRNA inhibits HIV production in infected primary CD4 cells. (a,b) HIV-infected CD4 T cells (~85% infected by p24 staining), treated with gag siRNA or GFP siRNA with no delivery agent or complexed with F105 or F105-P, were analyzed 2 d later for viral replication by intracellular p24 staining (a) and by p24 Ag ELISA of culture supernatants (b). The env-specific antibody by itself (see F105-P + irrelevant GFP siRNA and F105 + gag siRNA conditions) reduced HIV replication modestly (~25–28%) because of its viral neutralization activity, whereas F105-P-delivered gag siRNA reduced viral production by ~58% (by p24 staining) or by ~77% (ELISA) at the highest dose. These cells are resistant to lipid-mediated siRNA transfection and no inhibition of HIV was observed in transfected controls (data not shown).

tumor cells, then any gene required for normal cell growth or survival could in principle be targeted. Previously identified siRNAs against *c-myc*, *MDM2* and *VEGF*, alone and in combination, were tested for their ability to downregulate gene expression and reduce gp160-B16 cell proliferation *in vitro* (Fig. 4). Two siRNAs directed against *c-myc* were effectively and specifically loaded only into HIV env-expressing B16 cells to reduce *c-myc* mRNA, as assessed by quantitative RT-PCR (Fig. 4a) and *c-myc* protein, as assessed by flow cytometry (Fig. 4b). F105-P and *c-myc* siRNAs had no effect on B16 cells not expressing HIV env. When both *c-myc* siRNAs were loaded via F105-P into gp160-B16 cells, tumor cell proliferation was reduced in a dose-dependent manner, to a maximum threefold suppression at siRNA concentrations >100 nM (Fig. 4d). Proliferation was reduced to a similar extent at saturating siRNA concentrations of ~100 pmol. Similar gene silencing was obtained by delivering *VEGF* and *MDM2*

siRNAs (Fig. 4c, data not shown), but *VEGF* siRNA had only a modest effect on inhibiting *in vitro* tumor cell growth (Fig. 4e,f). However, by blocking angiogenesis, *VEGF* siRNA might have more of an effect *in vivo*. A control siRNA targeting the putative tumor suppressor pp32 (ref. 9) though effective at silencing gene expression, did not inhibit, but may have slightly enhanced, tumor growth (data not shown). Combining the siRNAs targeting *c-myc* and *MDM2* or all three genes led to the greatest inhibition of gp160-B16 cell proliferation. Tumor growth was inhibited sevenfold by siRNAs targeting all 3 genes, compared to threefold inhibition by combining the two siRNAs targeting *c-myc* or twofold inhibition or less by administration of each of the siRNAs against *MDM2* or *VEGF*, alone. (Fig. 4g). Moreover, none of these siRNAs delivered by F105-P had any significant effect on the growth of B16 tumor cells not expressing HIV env (Fig. 4h).

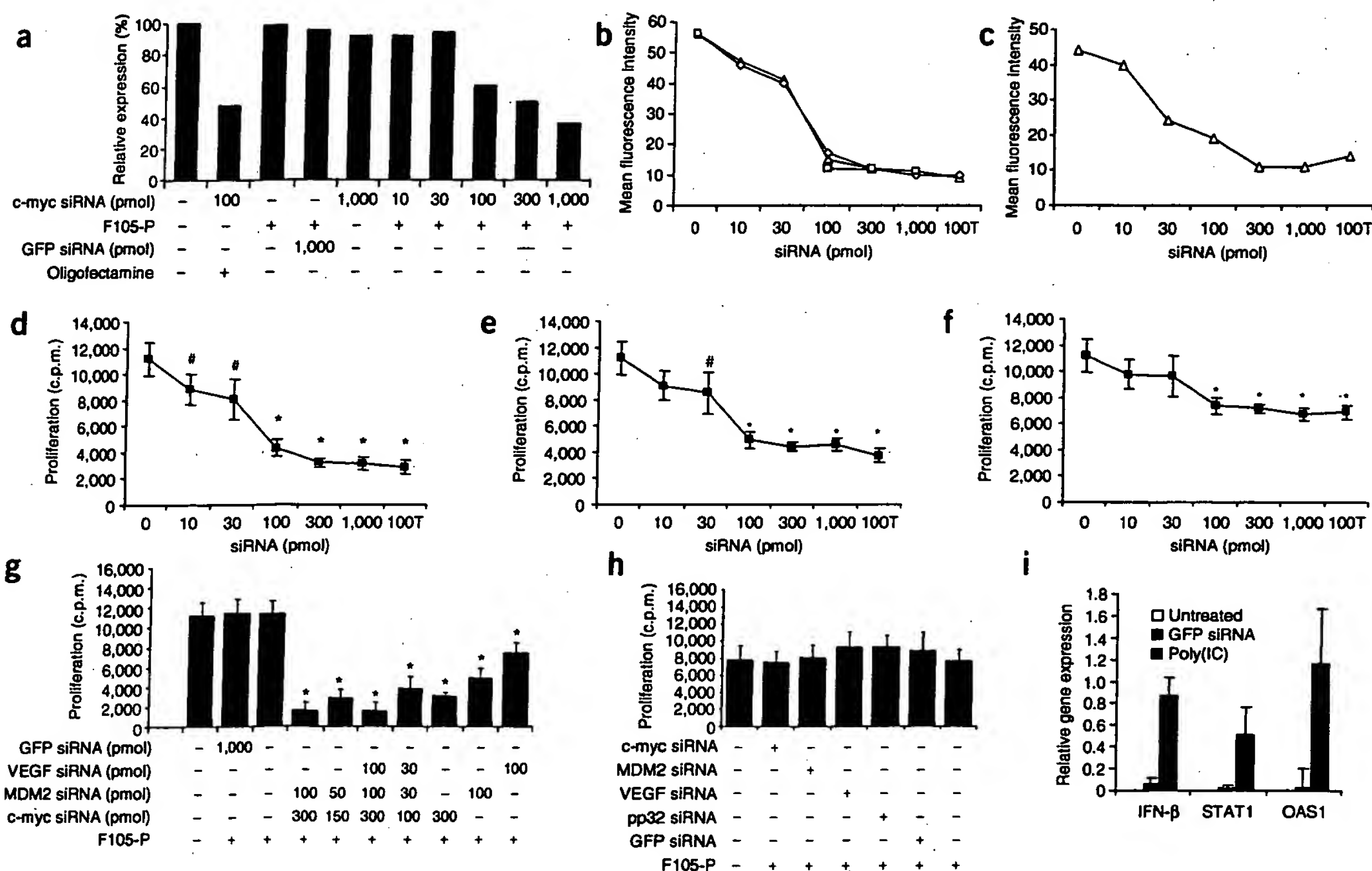


Figure 4 F105-P delivers *c-myc*, *MDM2* and *VEGF* siRNAs, silences gene expression, and inhibits tumor proliferation only in B16 melanoma cells expressing HIV env. (a–c) gp160-B16 were treated with increasing concentrations of two siRNAs directed against *c-myc* (a,b) or an siRNA targeting *VEGF* (c). Gene expression was analyzed by quantitative PCR (a) or flow cytometry of permeabilized cells (b,c). In b and c delivery was via F105-P except in the points marked 100T, which were via transfection of 100 pmol siRNA, a saturating concentration for transfection. Silencing in b was similar when similar concentrations of *c-myc* siRNA no. 1 (Δ), no. 2 (\diamond) or both (\square) were used. Controls in b and c also showed no reduction in mean fluorescence intensity by an irrelevant GFP-siRNA or in B16 cells not expressing gp160 (data not shown). Silencing requires gp160 expression on target cells and specific siRNA and is dose-dependent, reaching a plateau at about 100–1,000 pmol siRNA. Gene silencing was comparable when siRNAs were either delivered by F105-P or transfected with oligofectamine. (d–g) Proliferation of gp160-B16 cells treated with F105-P-delivered siRNAs directed against two *c-myc* sequences (d), *MDM2* (e), *VEGF* (f) or combinations of siRNAs (g). The effect of transfecting 100 pmol siRNA (100T) in (d–f) is comparable to F105 delivery of 100–300 pmol siRNA. Additional simultaneous controls performed for (d–f) showed no difference in proliferation with GFP-siRNA or when cells were treated with only siRNA or FP105 (data not shown). Combinations of siRNAs were more efficient at inhibiting tumor growth than single agents. (h) siRNAs complexed with F105-P had no effect on the growth of B16 cells not expressing HIV env. * denotes $P < 0.001$, whereas # denotes $P < 0.01$ as compared with untreated control. (i) IFN- β and the interferon response genes encoding STAT1 and OAS1, measured by quantitative RT-PCR, were not significantly induced one day after mock treatment (left bars, difficult to see above abscissa) or exposure to F105-P-delivered GFP-siRNA, but were induced by poly(I:C). Gene expression normalized to GAPDH mRNA.

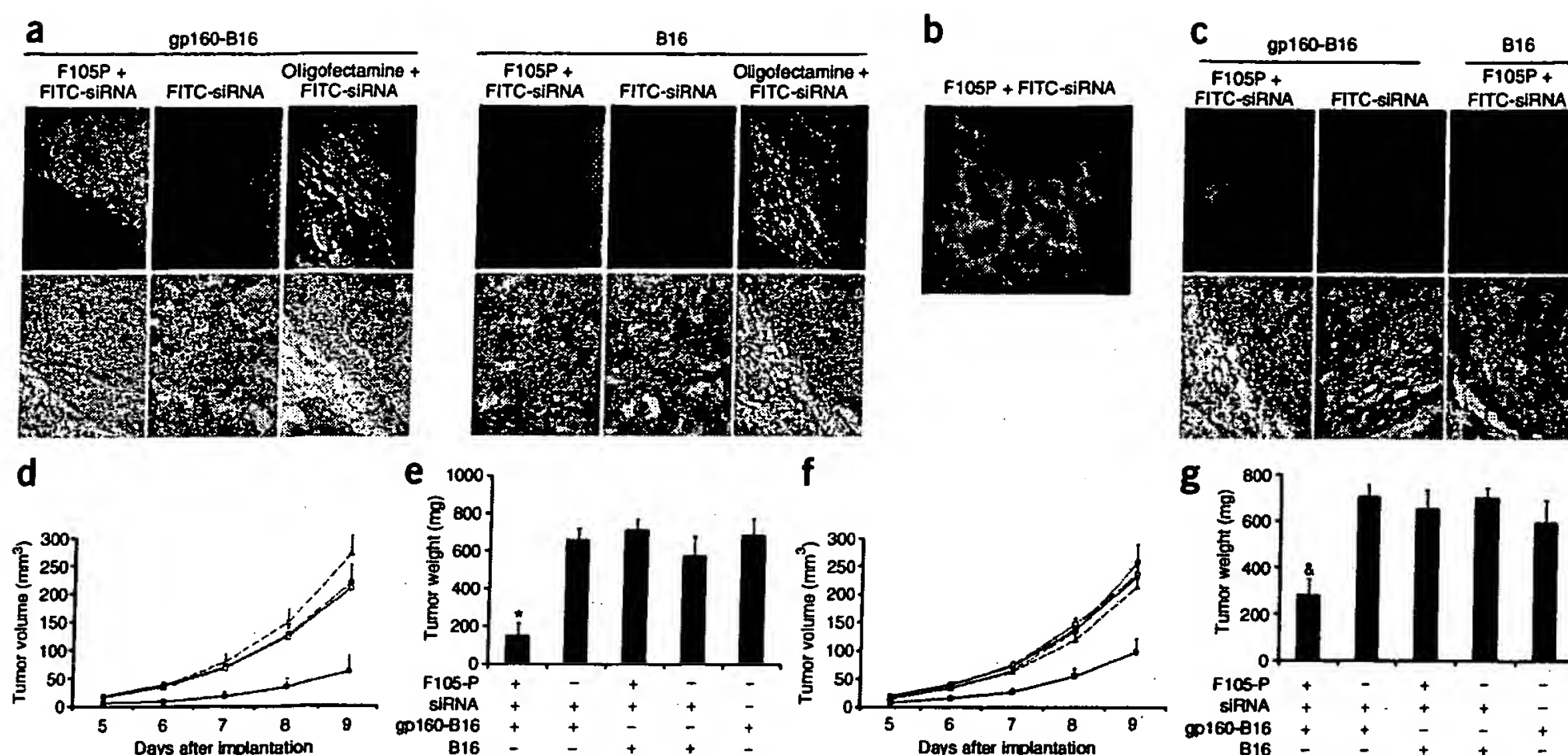


Figure 5 Intratumoral or intravenous injection of siRNAs complexed with F105-P delivers siRNAs only into env-bearing B16 tumors to suppress tumor growth. (a) Nine days after implanting gp160-B16 (left) or B16 melanoma cells (right) into the flanks of mice, FITC-siRNAs complexed with F105-P or oligofectamine were injected into the subcutaneous tumors. (b) A high power image of tumor cells injected with F105-P and FITC-siRNA shows fluorescent staining in the cytoplasm. (c) Alternatively, F105-P loaded with FITC-siRNA was injected intravenously. The tumors were harvested 12 h later for fluorescence microscopy (upper row) and hematoxylin and eosin staining (lower row). F105-P specifically delivers FITC-siRNA *in vivo* only into gp160-B16 tumors, but not into surrounding normal tissue or B16 tumors lacking env, whereas oligofectamine delivers FITC-siRNA into both tumor and neighboring tissues. Naked siRNAs do not efficiently get into any cells. Intratumoral injection is more efficient than intravenous injection. F105-P-delivered siRNAs targeting *c-myc*, *MDM2* and *VEGF* suppress the outgrowth of gp160-B16 tumors *in vivo*. (d–g) Mice were treated by intratumoral (d,e) or intravenous (f,g) injection on days 0, 1 and 3 after implanting B16 (dotted lines) or gp160-B16 cells (continuous and dashed lines) into the right flank in groups of 8 mice. A cocktail of siRNAs (*c-myc* no. 1 and no. 2, *MDM2* and *VEGF*) complexed with F105-P (■) or siRNAs alone (□) was injected. Mice mock-treated with PBS (Δ) served as a control. Tumor size (d,f) was measured daily and tumors were weighed (e,g) on day 9 when the animals were killed. The antitumor siRNAs suppressed tumor growth only when delivered by F105-P and only for tumors expressing HIV env. * denotes $P < 0.001$ as compared with PBS-injected controls. Injection of F105-P without siRNA provided no protection (data not shown).

siRNAs delivered by F105-P do not trigger interferon responses

Delivered siRNAs can potentially activate nonspecific inflammatory responses, which might cause toxicity, either because cytosolic double-stranded RNAs directly trigger an interferon response or do so indirectly via binding Toll-like receptors (TLR) that recognize RNA (TLR3, TLR7) on the cell surface or within endosomes. We therefore assayed by quantitative RT-PCR expression of interferon- β (IFN- β) and two key interferon responsive genes, 2', 5'-oligoadenylate synthetase (OAS1) and Stat-1, around the expected peak response time (24 h) in gp160-B16 cells that were either mock treated, exposed to irrelevant GFP siRNA delivered by F105-P or treated with the interferon inducer poly(I:C) (Fig. 4i). Although poly(I:C) induced the expression of all three genes, treatment with F105-P-complexed siRNA had no statistically significant effect on the expression of any of these genes. Therefore, F105 delivery of siRNAs does not appear to trigger potentially toxic IFN responses.

Specific systemic delivery of fluorescent siRNAs

The next step was to determine whether F105-P could specifically deliver siRNA into gp160-B16 cells *in vivo*. Therefore, we implanted gp160-B16 cells subcutaneously into the right flanks of syngeneic C57/BL6 mice and evaluated the efficiency of F105-P to deliver fluorescent siRNA. When naked FITC-siRNA was injected into the tumor tissue, the tumor cells did not take up siRNA efficiently. When FITC-siRNA mixed with oligofectamine was injected, the siRNA was taken up by the tumor and adjacent subcutaneous tissue. In contrast, F105-P

specifically delivered FITC-siRNA to gp160⁺ tumor cells, but not to adjacent tissue (Fig. 5a). On higher magnification, the fluorescent signal stained the cell membrane diffusely in the cytoplasm, but not in the nucleus of the tumor cells (Fig. 5b). F105-P delivery appeared specific because implanted B16 cells not expressing gp160 did not take up FITC-siRNA. Furthermore, about 30% of gp160-B16 cells, but none of the gp160⁻ B16 cells, took up FITC-siRNA when the F105-P-siRNA mixture was injected intravenously in a small (100 μ l) volume (Fig. 5c). No FITC-siRNA was taken up by the tumor after intravenous injection without F105-P.

siRNAs delivered by F105-P inhibit melanoma growth *in vivo*

To evaluate the therapeutic potential of antibody-mediated siRNA delivery, we injected F105-P complexed with a mixture of siRNAs against *c-myc*, *MDM2* and *VEGF* either directly into the tumor or intravenously on days 0, 1 and 3 after implanting 5×10^6 B16 or gp160-B16 tumor cells subcutaneously into the flanks of mice in groups of 8. Tumor size was measured beginning on day 5 when the tumors became palpable, and the tumors were weighed when mice were killed on day 9 (Fig. 5d–g). The gp160-B16 tumors were significantly smaller in mice treated with intratumoral or intravenous injection of F105-P-siRNA complexes when compared to those treated with siRNAs alone. Mice treated with just F105-P had similar size tumors as mice treated with PBS had (data not shown). Intratumoral injection was somewhat more effective than systemic delivery. As expected, growth of B16 tumors lacking gp160 expression was

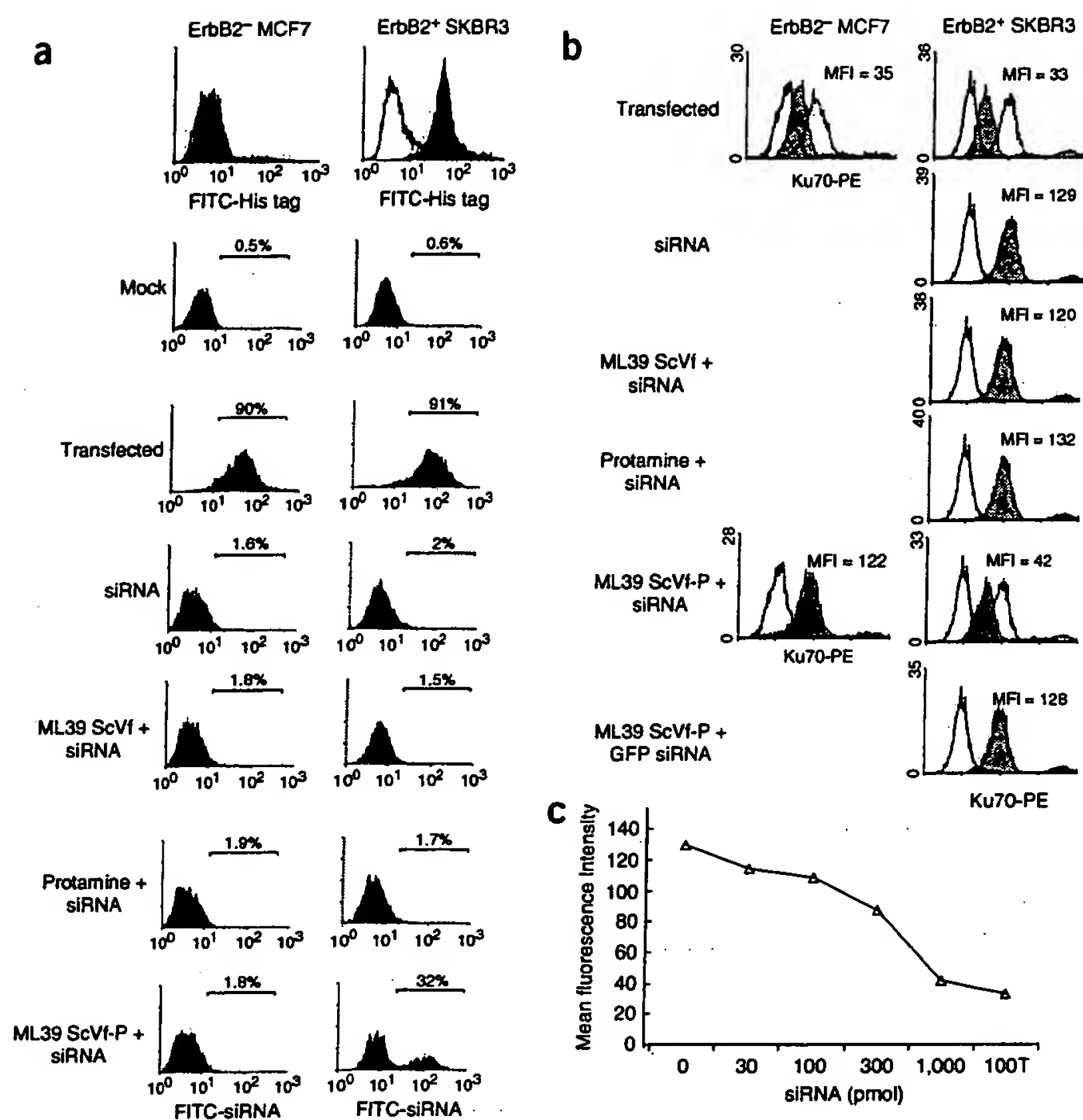


Figure 6 A single-chain antibody fragment against ErbB2 fused to a protamine fragment specifically and effectively delivers siRNAs only to ErbB2⁺ breast cancer cells. (a) The single-chain ErbB2 antibody protamine fusion protein ML39 ScVf-P binds to ErbB2⁺ SKBR3 cells, but not to ErbB2⁻ MCF7 cells (top row). Binding of the fusion protein is detected with His-tag antibody (red); isotype control-stained cells shown in white peak. ML39 ScVf-P delivers 100 pmol FITC-siRNA to 32% of SKBR3 cells but does not transduce MCF7 cells. The unmodified antibody, protamine alone or medium does not deliver FITC-siRNA. FITC-siRNA is introduced into both cell lines by transfection. (b) Delivery of Ku70-siRNA by ML39 ScVf-P reduces Ku70 expression only in ErbB2⁺ cells. In the flow plots, the white histogram represents isotype antibody-stained cells; the red histogram, mock treated cells; and the grey histogram cells, treated as indicated. Transfection of Ku70 siRNA equivalently reduces Ku70 expression in MCF7 cells (left) and SKBR3 cells (right). Delivery of Ku70 siRNA (1,000 pmol) by ML39 ScVf-P, but not using control proteins or ML39 ScVf-P plus GFP siRNA, silences Ku70. MFI, mean fluorescence intensity. (c) Dose response curve for Ku70 silencing using ML39 ScVf-P delivery. Ku70 MFI is shown. To achieve silencing comparable to that achieved with transfected siRNA (100 pmol, 100T) requires about 1,000 pmol of ML39 ScVf-P-delivered siRNA.

unaffected by F105-P-siRNA treatment and neither B16 nor gp160-B16 tumors were reduced by injection of naked siRNAs. Therefore, F105-P was able to deliver siRNAs specifically to env-expressing tumor cells to suppress tumor growth even when administered systemically.

Delivery by single-chain antibody fused to protamine

To determine whether antibody-mediated delivery of siRNAs could be used to target other cell-surface molecules besides gp120, we expressed a fusion protein, in baculovirus, composed of a single-chain antibody fragment (ML39 ScVf) that recognizes the receptor ErbB2, expressed on many breast cancer cells, fused at its C terminus to a fragment of protamine corresponding to amino acids 8–29 (ML39 ScVf-P)¹⁰. ML39 ScVf-P has previously been shown to introduce plasmid DNA only into ErbB2-expressing (ErbB2⁺) cells¹⁰. Because the fusion protein was retained within insect cells, it was extracted in guanidine HCl and refolded by gradual renaturation. Nonetheless, the yield of the single-chain antibody fusion protein from infected insect cells was much greater than the yield of F105-P expressed in mammalian cells. ML39 ScVf binds to ErbB2⁺ SKBR3 cells but not to ErbB2-negative (ErbB2⁻) MCF7 breast cancer cell lines (Fig. 6a). We first compared the ability of ML39 ScVf-P to introduce FITC-siRNA into SKBR3 versus MCF7 cells (Fig. 6a). ML39 ScVf-P delivered FITC-siRNA into 32% of ErbB2-expressing cells, but did not transduce ErbB2⁻ cells above background. There was no delivery without a carrier protein or by using the single-chain unmodified antibody or protamine alone. Because delivery was specific, we next looked at silencing of Ku70 by ML39 ScVf-P mediated delivery of siRNA targeted against Ku70 (Fig. 6b,c). Silencing of Ku70 occurred only

in ErbB2⁺ SKBR3 cells, not in MCF7 cells, and required the fusion protein and Ku70 siRNA since protamine or ML39 ScVf mixed with Ku70 siRNA or ML39 ScVf-P mixed with irrelevant EGFP siRNA had no effect on Ku70 expression. The dose response for silencing by ML39 ScVf-P plateaued at approximately 1,000 pmol of siRNA, about three- to tenfold more than is necessary for effective silencing with either transfection or F105-P delivery. Further work is needed to determine whether this could be improved by using full-length protamine (51 amino acids) fused to the single-chain antibody or optimizing the renaturation conditions or the binding ratio for siRNA.

DISCUSSION

We used HIV envelope protein as a model receptor for targeted delivery of siRNAs via an antibody Fab fragment fused to protamine. Delivery was specific to env-bearing cells both *in vitro* and *in vivo* and systemic delivery was possible by conventional intravenous administration. We introduced siRNAs into hard-to-transfect primary CD4 T cells and suppressed HIV production in already infected cells. Targeted delivery of siRNAs should raise the therapeutic index for siRNAs, reduce the amount of drug required and minimize concerns about off-target effects.

This proof of principle study lays the foundation for further improvements. As we showed for ErbB2, the fusion protein can be modified by replacing the Fab fragment with a single-chain antibody. The antibody could also be replaced by a cell-surface receptor ligand. In principle, full-length protamine or nucleic acid-binding protamine fragments or other nucleic acid-binding peptides could be fused to the

targeting moiety at either the N or C termini of the antibody or ligand. Other expression systems could also be used to produce the fusion protein. We presented preliminary evidence that the antibody fusion protein complexed with siRNA does not induce interferon² nor activate other nonspecific inflammatory responses⁴ when administered *in vitro*. This needs to be studied more carefully and verified *in vivo*. However, we did not observe any obvious toxicity or inflammatory infiltrate in our *in vivo* tumor model.

Pharmacokinetics of fusion antibody-delivered siRNAs remains to be determined. However, the fusion protein-complexed siRNAs will likely have a favorable half-life compared to unmodified siRNAs. Filtration of naked siRNAs by the kidney is the rate-limiting factor responsible for the short *in vivo* half-life of unmodified siRNAs. The estimated size of the complex (1 molecule of V_HC_{H1} (233 aa)-protamine (51 aa), 30 kDa; 1 molecule of V_KC_K, 28 kDa; 6 molecules of siRNA, 6 × 7,000 Da) is 100 kDa, well above the cut-off for kidney filtration. The fusion protein-siRNA complex is not likely to form particles that would be trapped in reticuloendothelial cells, such as tissue macrophages and dendritic cells, in filtering organs like the lung and spleen and interfere with systemic delivery. In fact no fluorescent siRNA uptake was noted in these organs (data not shown). Unmodified siRNAs have an *in vitro* serum half-life of ~1 h due to endogenous RNase activity¹¹. Binding to the fusion protein may protect complexed siRNAs from plasma RNases, but this needs to be determined. Chemical modification of the siRNA in the complex should reduce vulnerability to serum degradation, but whether modifications would enhance *in vivo* efficacy is uncertain, since chemical modifications appear to come at the price of efficiency for intracellular silencing¹².

Because siRNA transport required gp160 expression, our delivery strategy targeted antiviral siRNAs specifically into cells actively replicating HIV-1. Whether this would provide a feasible or optimal method for using siRNAs as small-molecule antiretroviral drugs to target infected cells is hard to predict. More efficient silencing might be achieved using another shared cell-surface receptor on the principal types of HIV-infected cells (CD4 T cells and macrophages), such as the HIV coreceptors CCR5 or CXCR4. Since our targeting strategy is flexible, the HIV-env antibody component of the fusion protein may be replaced by a specific antibody to a chemokine receptor or by the receptor ligand, such as a chemokine or chemokine analog. This would target cells at an earlier stage to prevent infection. However, specific delivery to already infected cells could be used to silence essential genes required for cell survival to eliminate infected cells without harming normal cells. Because siRNAs do not need to be covalently coupled to the antibody-fusion protein, the same reagent can be flexibly used to deliver changing mixtures of different siRNAs. Targeting multiple viral and/or host genes using cocktails of siRNAs could likely improve suppression of HIV infection over what we achieved by just targeting HIV *gag*¹³.

In this study we used an artificial system to target melanoma cells by transfecting them to express HIV env. This delivery strategy could be modified to target any of a variety of cells via different types of cell-surface receptors. Specific tumor markers, often indicators of a poorly differentiated state or of lineage commitment, have been identified for many human tumor cells. Examples include c-erbB2 (Her2) on some breast cancer cells¹⁴, the EBV-encoded LMP proteins on nasopharyngeal carcinoma or surface immunoglobulin on B cell lymphomas. However, there is some likelihood that tumors might be able to down-modulate expression of any particular cell-surface receptor to escape from therapy. A judicious choice of receptor to target (such as the receptor for a growth factor required for tumor proliferation, that

is, IL-6R on myeloma cells or IL-2R on T-cell lymphoma cells) might reduce the chance of escape. Normal cells, whose functions need to be regulated, could also be targeted by this method. Examples might be T lymphocytes in autoimmune disease, dendritic cells or macrophages during inflammatory diseases or hepatocytes for hypercholesterolemia. The latter have been recently targeted *in vivo* by systemic administration of a chemically modified siRNA covalently linked to a cell-receptor ligand (cholesterol binding to the apoB receptor)¹².

The trafficking pathway of F105-P-delivered siRNAs into the cytoplasm remains to be understood. The most likely pathway following cell-surface receptor binding is clathrin-mediated endocytosis. The efficiency of silencing compared to transfection suggests that if siRNAs are endocytosed, they are stable in the harsh endosomal milieu. How the siRNAs would exit from endosomes to the cytoplasm is unclear, but imaging (Fig. 6c) and silencing clearly show trafficking to the cytosol. A cell biology study to follow the delivery of fluorescent siRNAs is needed.

Overcoming the delivery obstacle is the greatest barrier for using siRNAs as small-molecule drugs for most indications (reviewed in¹). Although local delivery is possible via lipid-based methods, particularly at mucosal surfaces such as the skin or lung^{15–17}, (and as demonstrated here using oligofectamine for intratumoral delivery), systemic delivery is more challenging. The hydrodynamic injection method used in the first studies of *in vivo* protection from disease by siRNAs^{6,18,19} is not practical for human use. For some indications, local injection into the vein draining an organ may provide an alternate approach that could be used in humans^{20,21}. Approaches that have been reported to work *in vivo* include complexes with the polymer atelocollagen²², polyethyleneimine containing nanoparticles, lipid complexes, and liposomes^{23–25} and covalent linkage to cholesterol to target the liver¹². However, these methods are not cell-type specific. Silencing using antibody-mediated delivery is highly efficient, requiring about 15-fold less siRNA than was needed for cholesterol conjugated siRNA silencing *in vivo*¹⁰. Because our method does not involve covalent linkage of siRNAs or specialized chemistry, it has the advantage of flexibility, allowing easy preparation and evaluation of varying siRNA mixtures with the same delivery reagent. The method can be readily adapted to target almost any cell type. Moreover the method is readily accessible for *in vivo* testing by academic laboratories. Immunoliposomes that have been reported to deliver siRNA-encoding plasmids to glioma cells might also be adapted for cell-specific targeting of siRNAs²⁶. The method that works best *in vivo* may need to be tailor-made for the target cell and disease indication. Direct comparisons of different delivery approaches will be required to choose amongst possible strategies as they become available. However, this study and a recent report¹² suggest that the delivery obstacle can be overcome.

METHODS

siRNAs. siRNAs were synthesized using 2'-O-ACE-RNA phosphoramidites (A4 grade, Dharmacon Research). siRNAs directed against *fas* and *EGFP* were as previously described⁶. The sense and anti-sense strands of siRNAs were:

c-myc no. 1 (ref. 27): 5'-GAACAUCAUCAUCCAGGAC-3' (sense);

3'-CUUGUAGUAGUAGGUCCUG-5' (antisense);

c-myc no. 2 (ref. 27): 5'-ACUCGAACAGCUUCGAAAC-3' (sense);

3'-UGAGCUUGUCGAAGCUUUG-5' (antisense)

VEGF (ref. 28): 5'-CGAUGAAGCCUGGAGUGC-3' (sense);

3'-GCACUCCAGGGCUUCAUCG-5' (antisense);

MDM2 (ref. 26): 5'-GCUUCGGAACAAGAGACUCdTdT-3' (sense);

3'-dTdTGGUUGUGACGAAUGCGAAU-5' (antisense); and

pp32 (ANP32A) (ref. 29): 5'-AAGAAGCUUGAAUUAAGCGdTdT-3' (sense);

3'-dTdTUUCUUCGAACUUAUUCGC-5' (antisense)

Ku70: 5'-ACGGAUCUGACUACUCACUCAdTdT-3' (sense);

3'-dTdTUGCCUAGACUGAUGAGUGAGU-5' (antisense)

Fluorescent siRNAs directed against CD4 labeled with FITC at the 5' end of the sense strand as described⁶ were from Dharmacon.

Cell lines. COS, B16, Jurkat, SKBR3 and MCF7 cells (ATCC) were grown in RPMI1640 supplemented with 10% fetal bovine serum. HeLa cells stably expressing EGFP (HeLa-GFP) were previously described³⁰. Culture supernatants from COS cells stably transfected with pCMV-F105-P⁸ and grown in 500 ml rolling bottles at 37 °C were purified using protein L-agarose as described⁸. Stable transfectants of B16 cells expressing HIV env were produced using pcDNA3.1-EAC-1 (ref. 31) by G418 selection and single-cell cloning.

F105-P binding assay. To evaluate the binding capacity of F105-P, 200 pmol FITC-siRNA was added to dilutions of F105-P previously complexed to anti-protamine (Pharmingen)-coated protein A/G beads (Pierce). After overnight incubation at 4 °C and thorough washing, we determined absorbance at 488 nm and plotted it against a standard curve. Background binding to beads in the absence of F105-P was negligible.

siRNA delivery. The indicated siRNAs were mixed with protamine, F105, F105-P, ML39 ScFv, ML39 ScFv-P or PBS at a molar ratio of 6:1 (siRNA concentration, 300 nM) in PBS for 30 min at 4 °C before adding to cells. Nonadherent cells (4×10^5 cells in 400 μ l cell culture medium) were treated in 24-well plates. HeLa-GFP and B16 cells were similarly treated at ~75% confluency in 800 μ l in 6-well plates. For controls, cells were transfected with oligofectamine (Invitrogen) or TransIT-siQUEST (Mirus) following the manufacturers' protocol. Cells were analyzed for gene expression, HIV infection or proliferation 2 d after siRNA treatment.

HIV infection, transfection and detection. CD4 T cells, isolated from normal donor PBMCs by selection with CD4 immunomagnetic beads (Miltenyi Biotec), were stimulated with phytohemagglutinin (4 μ g/ml, Difco) for 4 d and infected with HIV strain IIIB (NIH AIDS Reagent Repository) at a multiplicity of infection (MOI) of 0.1. Seven days later, cells were treated with siRNAs as indicated. Similarly, Jurkat cells were infected with HIV strain IIIB at an MOI of 0.01 and treated with siRNAs 3 d later. HeLa-GFP cells in 6-well plates were transfected with λ HXB3 (NIH AIDS Reagent Repository) using an Effectene Transfection Kit (Qiagen) according to the manufacturer's protocol. Two days later, transfected cultures were treated with siRNAs as indicated and analyzed for HIV and GFP expression 2 d after treatment. GFP expression was analyzed by northern blot and by flow cytometry. Cells replicating HIV were identified by flow cytometry analysis of intracellular staining of permeabilized cells for RD1-conjugated anti-p24 as described¹³. Viral production was also assayed by p24 Ag ELISA (Perkin Elmer Life Science) of culture supernatants.

Flow cytometry. Trypsinized B16 cells were permeabilized using 0.1% Triton X-100 (Beckman Coulter) and stained with a rabbit anti-mouse c-myc or VEGF primary antibody (R&D Systems) followed by PE-labeled goat anti-rabbit secondary antibody (BD Pharmingen). Ku70 expression in breast cancer cell lines was similarly analyzed using PE-conjugated Ku70 antibody (Santa Cruz). Flow cytometry was performed on a FACScalibur with CellQuest software (Becton Dickinson).

Northern blot. Total RNA was harvested from treated HeLa-GFP cells using Trizol (Invitrogen Life Technologies) and analyzed by northern blot probed for GFP and β -actin as described³⁰. Delivery of siRNA into B16 cells was analyzed by modified northern blot designed to capture small RNAs efficiently as described¹³.

Interferon assay. gp160-B16 cells (1×10^6 /2 ml) were mock treated or treated with F105-P and GFP siRNA (300 pmol) or 5 μ g/ml poly(I:C). After 24 h RNA was isolated and analyzed by quantitative RT-PCR for induction of IFN or interferon responsive genes as described below.

Quantitative PCR. Total RNA (1 μ g) isolated with Trizol was reverse transcribed using Superscript III (Invitrogen) and random hexamers, according to

the manufacturer's protocol. Real-time PCR was performed on 0.2 μ l of cDNA, or a comparable amount of RNA with no reverse transcriptase, using Platinum Taq Polymerase (Invitrogen) and a Biorad iCycler. SYBR green (Molecular Probes) was used for the detection of PCR products. All reactions were done in a 25- μ l reaction volume in triplicate. Primers for mouse c-myc and GAPDH are:

GAPDH-forward 5'-TTCACCAACCATGGAGAAGGC-3',

GAPDH-reverse 5'-GGCATGGACTGTGGTCATGA-3',

c-myc-forward 5'-CCCCTGGTGCTCCATGAG-3',

c-myc-reverse 5'-TCCTCCTCAGAGTCGC-3'.

STAT1-forward 5'-TTTGCCCACTCGAGCTCCTG-3'

STAT1-reverse 5'-GGGTGCAGGTTCGGGATTCAAC-3'

OAS1-forward 5'-GGAGGTTGCAGTGCCAACGAAG-3'

OAS1-reverse 5'-TGGAAGGGAGGCAGGGCATAAC-3'

Interferon β -forward 5'-CTGGAGCAGCTGAATGGAAAG-3'

Interferon β -reverse 5'-CTTGAAGTCCGCCCTGTAGGT-3'

PCR parameters consisted of 5 min of Taq activation at 95 °C, followed by 40 cycles of PCR at 95 °C \times 20 s, 60 °C \times 30 s, and 69 °C \times 20 s. Standard curves were generated and the relative amount of target gene mRNA was normalized to GAPDH mRNA. Specificity was verified by melt curve analysis and agarose gel electrophoresis.

Cell proliferation. ³H-thymidine (1 μ Ci) was added for 6 h to treated B16 or gp160-B16 cells (2×10^4) in microtiter wells. Cells were harvested and analyzed by scintillation counting using a Top Count microplate reader (Packard).

Tumor implantation and treatment in mice. All animal experiments were approved by the CBR Institute Animal Care and Use Committee. Female C57/BL6 mice, 8–10 weeks of age weighing 20–25 g, were purchased from Jackson Laboratories. To evaluate *in vivo* delivery of siRNA, B16 or gp160-B16, cells (2×10^6) were injected subcutaneously into the right flank. The day after the first detection of tumors (day 9), mice were injected either directly into their tumor or intravenously with 50 μ g of FITC-labeled siRNA in PBS or mixed with oligofectamine or F105-P. Mice were killed 16 h later and tumors were snap-frozen for cryosectioning. Distribution of FITC-siRNA in tumors and adjacent tissue was evaluated by fluorescence microscopy and hematoxylin and eosin staining of consecutive sections. For treatment studies, B16 or gp160-B16, cells (5×10^6 cells) were implanted subcutaneously into the right flank of groups of 8 mice on day 0, and tumors were detected by day 5. F105-P complexed with c-myc siRNAs no. 1 and no. 2, MDM2 siRNA and VEGF siRNA (80 μ g siRNA in an injection volume of 100 μ l) at a molar ratio of F105-P/total siRNA of 1:6 was injected either directly into the tumor or intravenously on day 0, 1 and 3 after tumor implantation. Tumor size was measured daily by calipers from day 5 until day 9 after implantation. The mice were killed on day 9 and the tumor dissected and weighed.

Anti-ErbB2 ML39 ScFv and ScFv-P. Baculovirus vectors expressing His-tagged anti-ErbB2 ML39 ScFv and ScFv-protamine fragment (amino acids 8–29) (ScFv-P) were expressed and purified as previously described¹⁰. Briefly, ML39 ScFv was purified by Ni⁺⁺ chromatography after ammonium sulfate precipitation of culture supernatants of SF9 cells infected with recombinant viruses expressing ML39 ScFv, generated using BaculoGold (Pharmingen). ML39 ScFv-P was similarly expressed from baculovirus, but extracted from SF9 cells using 6 M guanidine hydrochloride (GuanHCl) followed by Ni⁺⁺ chromatography in 6 M GuanHCl. The eluted protein was gradually dialyzed into PBS and then into PBS containing 5% glycerol, 0.5 M arginine, 1 mM EGTA and 1 mM glutathione in reduced and oxidized form. Both proteins were finally dialyzed into PBS containing 5% glycerol, concentrated and stored at –70 °C.

Delivery using ML39 ScFv-P. To detect fusion protein binding, SKBR3 and MCF7 cells were detached using enzyme-free cell dissociation buffer (Life Technologies), washed with PBS containing 5% FBS, and incubated (5×10^5 cells/ml) for 30 min at 4 °C with ML39 ScFv-P (1 μ g/ml) before further incubation with FITC-conjugated His tag monoclonal antibody (Babco) for 30 min. Fixed cells were analyzed by flow cytometry. For delivery and silencing experiments, the indicated proteins were incubated with FITC-siRNA (300 nM) or Ku70 siRNA (indicated amounts) for 30 min at 4 °C before adding to cells.

FITC-siRNA delivery was assessed 4 h after culture at 37 °C and Ku70 silencing was assessed 3 d later.

Statistics. All *in vitro* experiments were performed in triplicate, except for the proliferation assay, which was performed in octuplicate. The results are described as mean \pm s.e.m. Statistical analysis was performed by one-way analysis of variance (ANOVA) and comparisons among groups were performed by independent sample *t*-test or Bonferroni's multiple-comparison *t*-test.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grant AI-056900 (J.L.), a Leukemia and Lymphoma Society fellowship (D.C.), amFAR scholar award (S.-K.L.), and Harvard CFAR scholar award (D.P.). We thank Manjunath N., Dong Zhang and Lianfa Shi for useful suggestions.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

Received 24 January; accepted 27 April 2005

Published online at <http://www.nature.com/naturebiotechnology/>

1. Dykxhoorn, D.M. & Lieberman, J. The Silent Revolution: RNA interference as basic biology, research tool and therapeutic. *Annu. Rev. Med.* **56**, 401–423 (2005).
2. Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. & Williams, B.R. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834–839 (2003).
3. Jackson, A.L. *et al.* Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637 (2003).
4. Kariko, K., Bhuyan, P., Capodici, J. & Weissman, D. Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J. Immunol.* **172**, 6545–6549 (2004).
5. Bitko, V., Musiyenko, A., Shulyayeva, O. & Barik, S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* **11**, 50–55 (2005).
6. Song, E. *et al.* RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351 (2003).
7. Chen, S.Y., Khouri, Y., Bagley, J. & Marasco, W.A. Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody. *Proc. Natl. Acad. Sci. USA* **91**, 5932–5936 (1994).
8. Chen, S.Y., Zani, C., Khouri, Y. & Marasco, W.A. Design of a genetic immunotoxin to eliminate toxin immunogenicity. *Gene Ther.* **2**, 116–123 (1995).
9. Chen, T.H. *et al.* Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. *Mol. Biol. Cell* **7**, 2045–2056 (1996).
10. Li, X., Stuckert, P., Bosch, I., Marks, J.D. & Marasco, W.A. Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells. *Cancer Gene Ther.* **8**, 555–565 (2001).
11. Layzer, J.M. *et al.* *In vivo* activity of nuclease-resistant siRNAs. *RNA* **10**, 766–771 (2004).
12. Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**, 173–178 (2004).
13. Song, E. *et al.* Sustained Small Interfering RNA-Mediated Human Immunodeficiency Virus Type 1 Inhibition in Primary Macrophages. *J. Virol.* **77**, 7174–7181 (2003).
14. Slamon, D.J. *et al.* Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177–182 (1987).
15. Sorensen, D.R., Leirdal, M. & Sioud, M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol.* **327**, 761–766 (2003).
16. Zhang, X. *et al.* Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J. Biol. Chem.* **279**, 10677–10684 (2004).
17. Massaro, D., Massaro, G.D. & Clerch, L.B. Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287**, L1066–L1070 (2004).
18. McCaffrey, A.P. *et al.* Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol.* **21**, 639–644 (2003).
19. McCaffrey, A.P. *et al.* RNA interference in adult mice. *Nature* **418**, 38–39 (2002).
20. Hamar, P. *et al.* Small interfering RNA targeting Fas protects mice against renal ischemia-reperfusion injury. *Proc. Natl. Acad. Sci. USA* **101**, 14883–14888 (2004).
21. Contreras, J.L. *et al.* Caspase-8 and caspase-3 small interfering RNA decreases ischemia/reperfusion injury to the liver in mice. *Surgery* **136**, 390–400 (2004).
22. Minakuchi, Y. *et al.* Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing *in vitro* and *in vivo*. *Nucleic Acids Res.* **32**, e109 (2004).
23. Schiffelers, R.M. *et al.* Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **32**, e149 (2004).
24. Hassani, Z. *et al.* Lipid-mediated siRNA delivery down-regulates exogenous gene expression in the mouse brain at picomolar levels. *J. Gene Med.* (2004).
25. Ge, Q. *et al.* Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. USA* **101**, 8676–8681 (2004).
26. Zhang, Y. *et al.* Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin. Cancer Res.* **10**, 3667–3677 (2004).
27. Hung, L. & Kumar, V. Specific inhibition of gene expression and transactivation functions of hepatitis B virus X protein and c-myc by small interfering RNAs. *FEBS Lett.* **560**, 210–214 (2004).
28. Reich, S.J. *et al.* Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* **9**, 210–216 (2003).
29. Brody, J.R. *et al.* pp32 reduction induces differentiation of TSU-Pr1 cells. *Am. J. Pathol.* **164**, 273–283 (2004).
30. Stewart, S.A. *et al.* Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* **9**, 493–501 (2003).
31. Muthumani, K. *et al.* Novel engineered HIV-1 East African Clade-A gp160 plasmid construct induces strong humoral and cell-mediated immune responses *in vivo*. *Virology* **314**, 134–146 (2003).